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Coagulation factor VIIa: prohemostatic drug and biomarker for thrombosis

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Sustained pro-haemostatic activity of rFVIIa in plasma and platelets in non-bleeding pigs may explain the efficacy of a once-daily prophylaxis in humans

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Abstract

Background: Recombinant factor VIIa (rFVIIa) is registered for treatment of inhibitor-complicated haemophilia, and a once-daily prophylactic administration of rFVIIa is successful in reducing the number of bleeding events. This suggests that a single rFVIIa dose has a pro-haemostatic effect up to 24 hours (h), which is difficult to explain given its half-life of 2 h. **Methods:** In this study, six pigs received a 90 µg/kg rFVIIa bolus. Plasma was collected and platelets were isolated at various time-points up to 48 h, and analysed for FVIIa levels and associated haemostatic activity. **Results:** Elevated plasma FVIIa levels were detected up to 24 h post-administration (36 (32-56) mU/ml [median (interquartile range [IQR]), 24 h] vs 2 (2-14) mU/ml [baseline]). Corresponding prothrombin time (PT) values remained shortened compared to baseline until 24 h post-administration (9.4 (9.3-9.9) seconds (s) [24 h] vs 10.5 (10.2-11.0) s [baseline], $p \leq 0.01$). The lag time in thrombin generation testing as well as clotting times in plasma-based assays were shortened up to 12 or 24 h post-administration, respectively (lag times 1.8 (1.7-2.1) minutes (min) [12 h] vs 2.3 (2.3-2.6) min [baseline], $p \leq 0.01$ and clotting times 3.8 (3.2-3.9) min [24 h] vs 5.2 (4.6-5.5) min [baseline], $p \leq 0.001$). Platelet FVIIa levels were elevated up to 48 h (7.7 (3.4-9.0) ng VIIa/mg actin [48 h] vs 2.5 (0.7-4.8) ng VIIa/mg actin [baseline]). **Conclusion:** Elevated and haemostatically active plasma and platelet FVIIa levels are detectable up to 24-48 h following rFVIIa administration in pigs. This prolonged pro-haemostatic effect of FVIIa may explain the prophylactic efficacy of a once-daily rFVIIa treatment.

Introduction

Recombinant factor VIIa (rFVIIa) has been shown in several clinical trials to be safe and effective for treatment of bleeding episodes in inhibitor-complicated haemophilia A and B, and has been registered for this purpose [1-5]. Infusion of rFVIIa results in enhanced local thrombin generation by both tissue factor-dependent and -independent mechanisms [6]. Enhanced thrombin generation by rFVIIa not only accelerates the formation of fibrin, but also results in enhanced platelet activation and inhibition of fibrinolysis [7,8]. These combined mechanisms may be responsible for the mechanism of action of rFVIIa in haemophilia.

Besides the use of rFVIIa in the treatment of bleeding episodes in inhibitor-complicated haemophilia, it has been repeatedly demonstrated that the prophylactic administration of rFVIIa is successful in reducing the number of bleeding events (reviewed in [[9,10]]). In 2007, Konkle et al. published the first and until now only randomised controlled trial of the prophylactic use of 90 or 270 µg/kg body weight rFVIIa [11]. For both dose administrations a reduction in the number of bleeding events (27-59%) was observed during and three months after the prophylaxis period compared to the pre-prophylaxis period. In 2012, Young et al. performed a retrospective observational study on the daily practice of clinicians worldwide when applying prophylaxis with rFVIIa [12]. The study confirmed a reduction of bleeding episodes of 50% by rFVIIa prophylaxis. rFVIIa has also been shown to be effective for prevention of spontaneous bleeding episodes and as prophylactic agent during surgery in patients with a congenital factor VII deficiency [13,14]. In these studies, rFVIIa also appeared to provide haemostatic efficacy for a time frame that is much longer than expected based on the plasma half-life.

Taken together, these clinical studies suggest that a single dose of rFVIIa has a pro-haemostatic effect up to 24 hours (h). The efficacy of rFVIIa during this time frame of prophylaxis is difficult to explain given its half-life of 2 h. The mechanism of action of prophylactically administered rFVIIa is still incompletely understood. The literature suggests two possible mechanisms which could explain the prolonged haemostatic effect of rFVIIa. First, the prolonged prophylactic effect of rFVIIa may be explained by the distribution of rFVIIa into the extravascular space, such as various tissues and bone joints [15-18]. This extravascular rFVIIa remains haemostatically active, and may be sufficient to raise the local haemostatic potential to such an extent that bleeding can be prevented.

A second possible mechanism regards internalisation and storage of rFVIIa by platelets [19], which may potentially prolong the rFVIIa half-life. Platelets that have taken up rFVIIa *in vitro* were shown to have increased haemostatic capacity as demonstrated by flow-based whole blood assays and thromboelastography [19].

To assess potential mechanisms by which rFVIIa may exert a prolonged haemostatic effect, we administered a 90 µg/kg body weight bolus administration of rFVIIa to non-bleeding pigs. During the time frame of prophylaxis, up to 48 h, plasma was collected and platelets were isolated and both analysed for FVIIa plasma and platelet levels and associated haemostatic activity.

Materials and methods

Study design

Six adult pigs, 80.5 (78.8-82.3) kg [median (interquartile range [IQR])], received a 90 µg/kg body weight bolus injection of rFVIIa (NovoSeven, Novo Nordisk, Bagsvaerd, Denmark), and were followed up for 24 h (n = 3) or 48 h (n = 3). The study was powered to detect a 25% difference in rFVIIa half-life in plasma compared to platelets, assuming an equal variance in plasma and platelet half-life. Blood was drawn into 3.4% sodium citrate (9:1, v/v) at baseline and at 5, 15, 30 min, 1, 2, 4, 6, 8, 12, 16, 24, and 48 h post-administration. All samples were successfully collected and processed, except for samples of two pigs at 24 h post-administration. Animal welfare was in accordance with institutional guidelines of the University of Groningen, and the experiment was approved by the local institutional Animal Care and Use Committee.

Plasma samples and platelet preparation

Blood samples were stored at room temperature for a maximum of 1 h after blood collection. Blood samples were centrifuged at 200 g for 15 min at room temperature to obtain platelet-rich plasma (PRP). To prevent platelet activation and aggregation during the platelet isolation, 10% v/v of acid citrate dextrose (ACD) and the prostaglandin analogue iloprost (final concentration of 10 ng/ml) were added to the PRP. Subsequently, the PRP was centrifuged at 500 g for 15 min at room temperature, and the platelet pellet was resuspended in Hepes-Tyrode buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 145 mM NaCl, 5 mM KCl, 0.6 mM NaH₂PO₄·H₂O, 1.0 mM MgSO₄·7 H₂O, 5 mM D-glucose, 0.1% w/v bovine serum albumin (BSA), pH 6). ACD and iloprost were added to prevent platelet activation during the following washing step. The platelets were centrifuged at 500 g for 15 min at room temperature, and the

platelet pellet was resuspended in a small volume of Hepes-Tyrode buffer. Platelet lysates were obtained by freeze-thawing the samples twice, and the samples were subsequently centrifuged at 20,000 g for 10 min at 4°C. Platelet-poor plasma (PPP) was obtained by double centrifugation at 2,000 g and 10,000 g both for 10 min at room temperature. The platelet lysates and PPP samples were snap-frozen and stored at -80°C until use.

Plasma FVIIa activity

Plasma FVIIa levels (FVIIa:C) were measured using a commercially available kit according to manufacturer's instructions (STACLOT VIIa-rTF, Diagnostica Stago, Asnieres, France). This assay is based on the use of recombinant soluble tissue factor (rsTF) which can no longer activate FVII to its activated form, while retaining the ability to serve as cofactor for the FVIIa-catalysed activation of FX. A one-phase exponential decay curve fitting was applied to calculate the FVIIa plasma half-life, using the GraphPadPrism software package (Graphpad Software, Inc., La Jolla, CA, USA).

Prothrombin time

The prothrombin time (PT) was measured on an automated coagulation analyser (Behring Coagulation System, BCS) using the manufacturer's reagents and protocols (Siemens Healthcare Diagnostics, Marburg, Germany).

Thrombin generation assay (TGA)

Plasma haemostatic potential was determined by *in vitro* thrombin generation using calibrated automated thrombography and the manufacturer's reagents and protocols (Thromboscope B.V., Maastricht, the Netherlands). Specifically, low PPP-reagent containing TF (final concentration 1 pM) and phospholipids (final concentration 4 µM) was used. The thrombograms were measured in a 96-well plate fluorometer (Fluoroskan Ascent Microplate Fluorometer, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Plasma-based microtitre plate clotting assay

Coagulation was studied by monitoring changes in turbidity during clot formation in a 96-well microtitre plate (Immulon 2HB flatbottom microtitre plates, Thermo Fisher Scientific Inc., Waltham, MA, USA). A mixture of TF (Innovin [Siemens Healthcare Diagnostics], final dilution 10⁵ times), CaCl₂ (final concentration of 10 mM) and phospholipid vesicles (final concentration of 10 µM) in a volume of 50 µl Hepes buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, pH7.4) was added to 50 µl of citrated

plasma in a microtitre plate, and thoroughly mixed. Clot formation was followed by monitoring changes in turbidity at 405 nm over time at 37°C in a VersaMax Microplate Reader using SoftMax Pro software (Molecular Devices, Sunnyvale, CA, USA). Clotting time was defined as the midpoint of clear-to-maximum-turbid transition.

Microtitre plate clotting assay to estimate FVIIa levels in platelet lysates

The FVIIa activity in platelet lysates was studied by the microtitre plate clotting assay as described in the previous paragraph with a few adaptations. In short, 5 µl of platelet lysate sample was added to 50 µl FVII-deficient plasma (Haematologic Technologies, Inc., Essex Junction, VT, USA). Coagulation was initiated by adding 50 µl of a mixture of TF, CaCl₂, and phospholipid vesicles in Hepes buffer to the plasma, and this was mixed thoroughly. Clot-formation was followed by monitoring changes in turbidity at 405 nm over time at 37°C and clotting times were determined as the midpoint of clear-to-maximum-turbid transition. A calibration curve of rFVIIa was used to convert clotting times to FVIIa concentrations, which were normalised for actin levels of corresponding samples. Platelet FVIIa levels in all samples were determined at two separate occasions and the mean of these two assays was used.

Actin levels of platelet lysate samples

Actin levels were determined as previously described [20] with minor adjustments. In short, 2 µg/ml of a monoclonal antibody to actin (MAB1501R, Millipore, Billerica, MA, USA) in phosphate buffered saline (PBS, 10 mM Na₂HPO₄, 3.2 mM KH₂PO₄, 120 mM NaCl, pH 7.2) was coated in a 96-well plate at 4°C overnight. The next day the plate was washed with 0.1% v/v Tween/PBS and subsequently blocked for 2 h with 3% w/v BSA/PBS. Samples were diluted in 3% w/v BSA/PBS and incubated for 1 h at 37°C. The plate was washed before addition of 800 ng/ml of a biotinylated chicken anti-actin antibody (MAB1501R, Chemicon (Millipore), biotinylation kit (Pierce, Rockford, IL, USA)) which was incubated for 1 h at 37°C. After washing, 1:200 streptavidin conjugated to HRP (Dako, Glostrup, Denmark) was added and incubated for 30 min at 37°C. After washing, TMB substrate (Sigma-Aldrich, Zwijndrecht, the Netherlands) was added and the reaction was stopped by adding 1 M H₂SO₄. Colour intensity was measured using a VersaMax Microplate Reader, and translated to actin levels using a calibration curve of known concentrations of actin (APHL-95, Cytoskeleton, Denver, CO, USA).

Statistical analysis

To evaluate differences between measurements at various time points post-administration and the pre-administration (baseline) measurement, a linear mixed effect model was used. This model takes the correlation between the repeated measurements within a single pig into account. P-values ≤ 0.05 were considered statistically significant. All analyses were performed using the statistical software package SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Plasma FVIIa levels remain elevated up to 24 h post-administration

When rFVIIa was administered to non-bleeding pigs, plasma FVIIa levels reached a maximum level at 5 min post-administration (30306 (29730-31248) mU/ml [median (interquartile range [IQR])]) and decreased to pre-administration levels over time with a half-life of 2 h (Figure 1). Elevated plasma FVIIa levels were detected up to 24 h post-administration (36 (32-56) mU/ml at 24 h vs 2 (2-14) mU/ml at baseline), although differences measure beyond 6 h did not reach statistical significance.

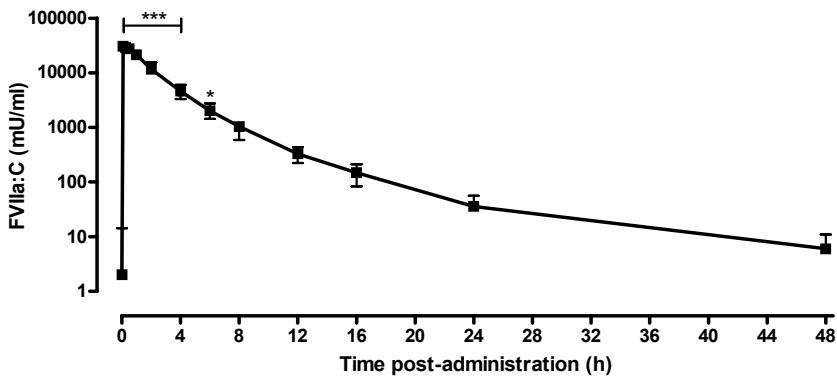


Figure 1 FVIIa levels of pig plasma samples at various time-points post-administration of a 90 $\mu\text{g}/\text{kg}$ body weight bolus injection of rFVIIa were measured using the STACLOT VIIa-rTF assay. FVIIa levels are expressed as mU/ml, in which 30 mU is equivalent to 1 ng FVIIa. Shown are medians, and error bars indicate IQRs. *** $p \leq 0.001$, * $p \leq 0.05$ vs baseline measurement.

Enhanced plasma haemostatic potential throughout the time frame of rFVIIa prophylaxis

To assess plasma haemostatic potential, plasma samples were analysed using a PT, *in vitro* thrombin generation, and a plasma-based microtitre plate clotting assay. Immediately after administration of rFVIIa, PT values decreased and remained shortened compared to baseline until 24 h post-administration (9.4 (9.3-9.9) s at 24 h vs 10.5 (10.2-11.0) s at baseline, $p \leq 0.01$), as shown in Figure 2. However, the clotting times remained shortened compared to baseline even at 48 h post-administration (10.0 (9.9-10.0) s at 48 h vs 10.5 (10.2-11.0) s at baseline), although the difference at the 48h time-point did not reach statistical significance.

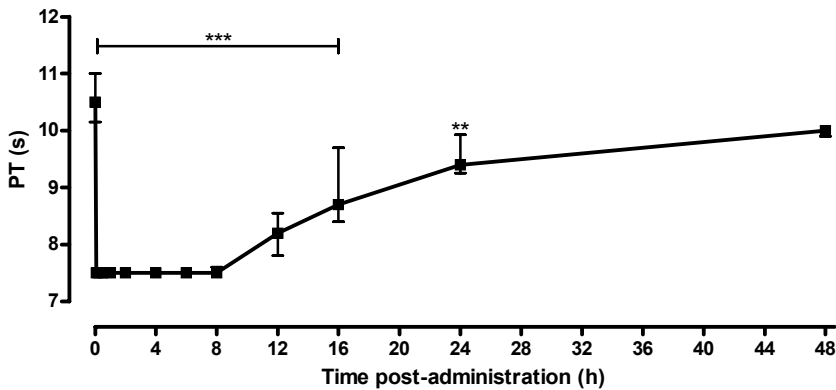


Figure 2 Prothrombin times of pig plasma samples at various time-points post-administration of a 90 $\mu\text{g}/\text{kg}$ body weight bolus injection of rFVIIa. Shown are medians, and error bars indicate IQRs. *** $p \leq 0.001$, ** $p \leq 0.01$ vs baseline measurement.

In addition, the plasma haemostatic potential was assessed by *in vitro* thrombin generation. Lag times derived from the thrombin generation curves are shown in Figure 3. In post-administration plasma samples, lag times were significantly shortened up to 12 h post-administration (1.8 (1.7-2.1) min vs 2.3 (2.3-2.6) min at baseline, $p \leq 0.01$), but only returned to pre-administration levels at 48 h.

Finally, plasma haemostatic potential was estimated by a plasma based microtitre plate clotting assay, of which the results are shown in Figure 4. Immediately after rFVIIa administration the clotting time decreased and remained shortened until 24 h post-administration (3.8 (3.2-3.9) min vs 5.2 (4.6-5.5) min at baseline, $p \leq 0.001$). However, the clotting times remained shortened compared to baseline even at 48 h post-administration

(4.3 (4.0-5.0) min at 48 h vs 5.2 (4.6-5.5) min at baseline), although the difference at the 48 h time point did not reach statistical significance.

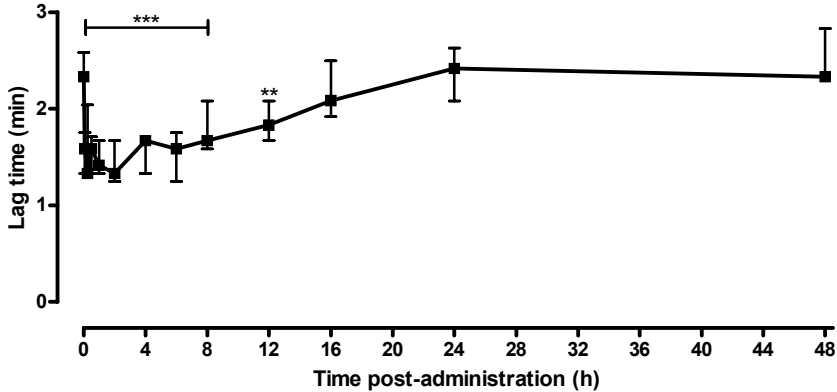


Figure 3 Pig plasma samples at various time-points post-administration of a 90 µg/kg body weight bolus injection of rFVIIa were tested in thrombin generation assays. Shown are medians, and error bars indicate IQRs. *** $p \leq 0.001$, ** $p \leq 0.01$ vs baseline measurement.

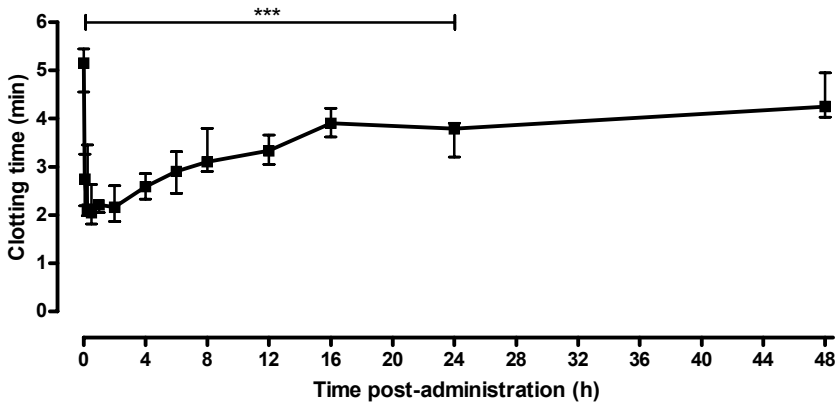


Figure 4 Pig plasma samples at various time-points post-administration of a 90 µg/kg body weight bolus injection of rFVIIa were tested in a plasma-based microtitre plate clotting assay. Clotting time was defined as the midpoint of clear-to-maximum-turbid transition. Shown are medians, and error bars indicate IQRs. *** $p \leq 0.001$ vs baseline measurement.

Elevated levels of functionally active rFVIIa in platelet lysates throughout the time frame of prophylaxis

Concentrations of FVIIa in platelet lysates were determined using a microtitre plate clotting assay using FVII-deficient plasma. Prior to administration of rFVIIa, detectable levels of FVIIa were present in pig platelets (2.5 (0.7-4.8) ng FVIIa/mg actin). Immediately after administration of rFVIIa, levels of FVIIa substantially increased to a maximum of 7.5 (5.5-19.7) ng FVIIa/mg actin as shown in Figure 5. FVIIa levels in the platelet lysates remained elevated compared to baseline levels throughout the experiment (up to 48 h post-administration; 7.7 (3.4-9.0) ng FVIIa/mg actin at 48 h vs 2.5 (0.7-4.8) ng VIIa/mg actin at baseline), although differences did not reach statistical significance.

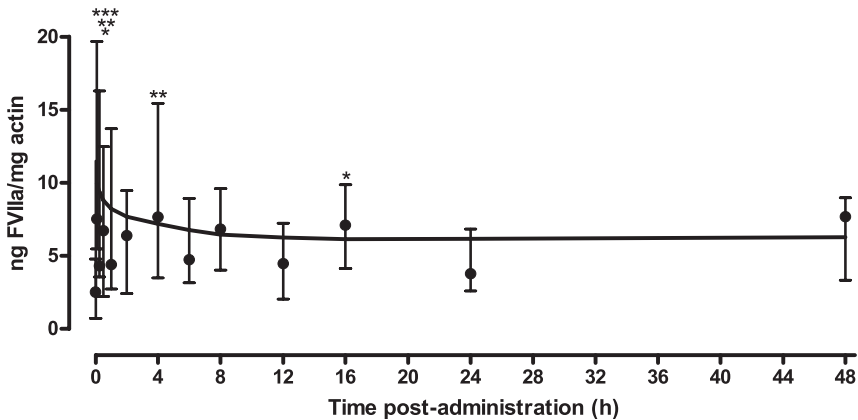


Figure 5 FVIIa levels measured in pig platelet lysate samples at various time-points post-administration of a 90 $\mu\text{g}/\text{kg}$ body weight bolus injection of rFVIIa. FVIIa levels were normalised for actin levels to correct for enumeration differences between samples. Shown are medians, and error bars indicate IQRs. *** $p \leq 0.001$ ($t = 5$ min), ** $p \leq 0.01$ ($t = 15$ min, 4 h), * $p \leq 0.05$ ($t = 30$ min, 1 h, 16 h) vs baseline measurement.

Discussion

The present study shows elevated levels of FVIIa in plasma and platelet lysates up to 24 or 48 h post-administration of a 90 µg/kg body weight bolus injection of rFVIIa in non-bleeding pigs. Small but detectable amounts of FVIIa are present in plasma at the end of the time frame of prophylaxis. Importantly, these low levels of FVIIa detected at the end of the time frame of prophylaxis are still haemostatically active as shown by three distinct functional tests. In addition, low but detectable levels of FVIIa were found in platelet lysates using a functional assay.

It has been reported previously that small amounts of FVIIa remain detectable in plasma for 20-24 h post injection in non-bleeding healthy human subjects. In addition it has been demonstrated that the results of clotting tests such as the PT remain shortened for this time frame up to 24 h after rFVIIa injection in these individuals [21,22]. Furthermore, FVIIa plasma levels appear elevated compared to baseline in patients with haemophilia at 12 h post-injection [23,24]. Also in these haemophilia patients who experience bleeding episodes, the PT was shortened compared to baseline up to 12 h after a 70 µg/kg infusion of rFVIIa [24]. However, the authors of these previous studies did not interpret these low levels of FVIIa which are still present hours after the infusion as being clinically relevant.

Lopez-Vilchez et al. showed the *in vitro* redistribution of rFVIIa into platelets [19], and our study is to our knowledge the first study to show an increase in FVIIa levels in platelet lysates following rFVIIa administration suggesting *in vivo* uptake of rFVIIa by platelets. The half-life of FVIIa taken up by platelets appears to exceed the plasma half-life, which means that the uptake of FVIIa by platelets protects FVIIa against clearance from the circulation. Although the platelet FVIIa levels are low, they may be of particular importance when plasma FVIIa levels are low at the end of the time frame of prophylaxis. At that point in time, local activation of platelets with concomitant release of platelet FVIIa may result in a locally elevated FVIIa concentration.

Taken together, we postulate that both the low levels of FVIIa circulating in the plasma and internalised rFVIIa in platelets hours after a bolus injection of rFVIIa are responsible for the prevention of bleeding episodes in inhibitor-complicated haemophilia. We propose that much lower plasma levels of rFVIIa are required for prevention of bleeding than is required for treatment of active bleeds. Although it has been well established that treatment of bleeding episodes in patients with haemophilia requires peak plasma levels of at least 8-12 nM (20-30 IU/ml) [25], the minimal dose of rFVIIa required for prevention of bleeding is unknown. However, the clinical efficacy of a once-daily

prophylactic regimen suggests that the FVIIa concentration required for prevention of bleeding may be orders of magnitude lower. Studies to substantiate this hypothesis will be difficult to accomplish in animal models given the lack of spontaneous joint and muscle bleeding, but studies in haemophilia patients may be feasible. Prophylactic studies in humans using continuous or semi-continuous infusion of a mini dose of rFVIIa resulting in low but detectable rFVIIa plasma levels would show whether our hypothesis is correct. Such a study may, for example, be achieved using subcutaneous infusion with an insulin pump. Subcutaneous administration of rFVIIa has been demonstrated to be feasible in haemophilia patients [26]. In case such a continuous or semi-continuous infusion of a mini dose of rFVIIa is as effective in preventing bleeding as a once-daily 90 $\mu\text{g}/\text{kg}$ dosage, this may lead to significant cost reductions.

Although we confirmed and extended previous data on the prolonged presence of rFVIIa in plasma, and are the first to demonstrate *in vivo* uptake of rFVIIa by platelets, our approach obviously has limitations. First, we studied effects of human rFVIIa in a pig model, and although the clearance of plasma rFVIIa in terms of half-life appears very similar in pigs compared to humans, effects of species incompatibility on our analyses cannot be fully ruled out. Second, we studied haemostatic effects of rFVIIa in haemostatically competent and non-bleeding animals. Although we observed clear effects of rFVIIa infusion on thrombin generation lag time and clot formation time, rFVIIa did not enhance total thrombin generation (data not shown), which is likely already optimal in pigs with a fully competent haemostatic system. Studies on the haemostatic effects of low plasma concentrations of rFVIIa in models of haemophilia are required, and effects in haemophilia models in which spontaneous bleeding occurs are desirable. Third, in this study we did not assess the in literature mentioned potential other mechanism which may explain the prophylactic efficacy of a once-daily rFVIIa regimen (accumulation of rFVIIa in extravascular compartments), but this possibility will be explored and reported elsewhere. Finally, the number of animals used in this study was limited which necessitates cautious interpretation of the statistical analyses.

In conclusion, we propose that the low, but haemostatically active, FVIIa plasma and internalised rFVIIa platelet levels present hours after a 90 $\mu\text{g}/\text{kg}$ infusion could explain the haemostatic efficacy of a once-daily prophylactic regimen in humans. Further study is required to elucidate whether the success of a once-daily prophylactic regimen of rFVIIa is due to a low plasma maintenance level of FVIIa which is sufficient to prevent bleeding in patients with inhibitor-complicated haemophilia, or whether other mechanisms are involved.

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